The Effects of Centrifugation on Equine Spermatozoa

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Abstract

The business of reproductive physiology for the horse is very different from that of other farm animals. Breed associations are just beginning to allow modem technologies such as cooled semen to be employed in the industry. As these practices are allowed, research needs to go into discovering the best possible techniques to make the industry efficient

This experiment examined the sperm motility of three stallions under three separate conditions: centrifuged and diluted with extender, centrifuged with the removal of seminal plasma then diluted and diluted with no other alterations. These conditions were created using four different semen extenders to reduce individual variability as well as variability between individuals.

The results demonstrated that overall centrifugation had no detrimental effects on the motility of the semen either between subjects or for an individual ($p=0.4234$ and $p=0.4530$). There was also no statistically significant difference between extenders which contradicts some previous research done at Texas A&M university in 1997. There was a significant difference of motility over the four different time periods ($p<0.0001$) which is explained by the fact that the spermatozoa were dying as time progressed.

This research is helpful in that by knowing that centrifugation is not detrimental to sperm motility there is no need to find another method to remove seminal plasma. As more is found out about seminal plasma, the practice of its removal may be more highly recommended. As a suggestion for future research, the use of more stallions would be recommended as it is documented that stallions vary in their ejaculate ability.

Objectives

The primary goal of this project is to determine the effects of centrifugation at 10,000g's for 15 minutes on the percent motility of equine spermatozoa from three different stallions. Through the data taken, differences as a result of different extenders and variations within and between stallions was also analyzed.

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Introduction

As cooled, shipped semen becomes more prevalent in the business of equine reproduction, all aspects of the procedure must be studied and understood. One common practice is the centrifugation of the equine ejaculate to remove a majority of the seminal plasma. The seminal plasma varies between individual stallions and can be detrimental to the motility of the spermatozoa. This experiment serves to examine the effect that centrifugation has on the longevity of spermatozoa.

This experiment examined three stallions, resulting in twelve samples, and looked at the variation of motility in the sperm under three separate conditions: 1) centrifuged and diluted with extender 2) centrifuged with the removal of seminal plasma then diluted and 3) simply diluted. These conditions were performed using four different semen extenders as variability has been reported to exist between individuals and a certain types of extender.

Literature Review

In the past few years using cooled shipped semen for equine artificial insemination has become more popular. The advantages of cooled shipped semen are decreased cost of shipping mares to breeding farms, the availability of stallions many miles away to the mare owner and more widespread popularity of a stallion over a larger geographic area. The down side of cooled shipped semen is that is requires good maintenance of the mare's reproductive cycle and the issue of getting the semen to the breeder's farm in time.

Although the idea of cooled semen for artificial insemination is popular with many breeders, within the United States not all breed registries will accept foals produced by this method (1). The fear that breeders will cheat and use non-breed semen is the major reason for the reluctance of allowing this practice. Even among registries that allow artificial insemination, some, such as the thoroughbred industry still require the semen to be used immediately and on the farm where the stallion lives. However, with the advent of DNA testing, more associations are permitting the use of transported cooled semen for insemination on farms across the nation. In order to prevent fraud, DNA testing of the mare, stallion and foal are required before the foal will be recognized and registered in the breed. The two most prominent laboratories that do DNA testing are the University of California - Davis and the University of Kentucky in Lexington. As a result of these new methods the American Quarter Horse Association has approved cooled shipped semen for the year 1997 (13).

The fertility of cooled semen ranges from 0% to > 70% the upper range which is comparable to the 75% with the use of fresh semen proves that in the right hands, cooled semen is very effective (1). There are many factors that go into fertility rates using cooled

semen, including but not limited to: frequency of insemination with the cooled semen, concentration of sperm in extender, stallion variability in response to cooling, type of antibiotics in extender, as well as storage duration and temperature.

Many extenders have been tested for their ability to preserve equine semen. Some examples of extenders treated to preserve cooled semen are: home made egg yolk and glycerol added to non-fat dry skim milk-glucose extender (DSMG) (4), Colorado extender (3), EZ-Mixin, Kenny's, and modified INRA82 (9). The most successful of these without washing with media after centrifugation at 24 hours was the INRA82 which maintained motility of 61.1+-8.2 % after 72 hours as compared to 47.9+-6.8 % after 72 hours for EZ-Mixin (9). The modified INRA82 extender consists of glucose, lactose, raffinose, trisodium citrate dehydrate, potassium citrate, Hepes, penicillin, Gentamycin, water and HT skimmed milk (13).

In a 1997 study at the College of Veterinary Medicine at Texas A&M University, seven popular extenders were compared using the semen from three different stallions. The sperm was evaluated on the basis of six characteristics including: percentage of motile sperm, percentage of rapidly-motile sperm, mean curvilinear velocity, mean average path velocity, mean straight-line velocity and percentage of progressively motile sperm. The results concluded that Polymixin B yielded lower rates than other extenders, which included Ticarcillin, amikacin and Gentamycin. Amikacin provided the best environment for the sperm. None of these extenders were capable of inhibiting all bacterial growth especially of the *Enterobacteriae* family, however, Gentamycin and amikacin were at the top of the list for preventing bacterial growth. There were, however, some inter-stallion differences in response to the different extenders but the overall results are as summarized above (17).

The type of container used for cooling and shipping semen is also an important factor. A study in 1997 looked at the effectiveness of three of the most popular containers on the market today: the Equitainer, ExpectaFoal, and Equine Express (formerly called the Salsbro Box). All three were effective in cooling the semen at an acceptable rate (less than -1.0 degrees Celsius/min and storing it at a preferable temperature (approximately 5 degrees Celsius) for up to 24 hours. The Equitainer was the most successful at maintaining temperatures for longer periods of time, up to two days and is commonly used by The Ohio State University. It required 11 hours and fifteen minutes to obtain its lowest temperature and 53 hours to exceed 10 degrees Celsius when left in the container (10).

A traditional practice in artificial insemination with cooled semen is the shipment of two doses of semen one to be used on arrival and the other twenty four hours later. A 1997 study specifically looked at this practice to determine if there was any pregnancy rate difference in the insemination of the mare using both doses at once or at 24 hour intervals. The study included thirty-six mares to be inseminated by one stallion. Eighteen mares were inseminated with 500 million sperm cooled for 24-48 hours; the remaining eighteen were inseminated with 250 million sperm cooled for 24 hours and 24 hours later with 250 sperm cooled for 48 hours. The results concluded that the pregnancy rates were the same for both groups and that there is no advantage to waiting 24 hours to inseminate with the remaining half of the shipped semen (15).

There is variability among stallions in regard to successfully maintaining fertility in cooled shipped semen. It is not clear yet where the variability lies, however, once reasons for the variability are found, methods can then be used to found the maintenance of sperm fertility for all stallions. The quality of seminal plasma is one of the factors that is presently knowm to vary between stallions. Current studies are determining the effect of these

proteins and their characteristics among and between stallions. Centrifugation to remove the seminal plasma can and is done. This is usually done prior to the addition of the extender and cooling (1). Some studies have been done on seminal plasma removal, but the data have not been combined with the effects that centrifugation might have on the life of sperm in cooled shipped semen (3).

Knowing that there is seminal plasma variability between stallions, researchers are looking at the effect of seminal plasma on the mares' reproductive tract and characterizing the proteins found in the plasma. A 1997 review from the University of California-Davis stated that there is a factor in seminal plasma that allowes semen to remain viable in the female reproductive tract. Perhaps seminal plasma contains proteins that prevent premature capacitation of sperm as the mare's reproductive tract mounts an immune response to the semen by producing leukocytes, namely PMNs (12). However, there is variation in the response of mares to different stallions and that difference is believed to lay in the variatoins of seminal plasma.

Following this belief, Dr. Frazer et. al. of The Ohio State University did a 1995 study to characterize seminal plasma proteins from fourteen different stallions. They were able to isolate fourteen different proteins in the seminal plasma with sizes between 14 kDa and 120 kDa. None of the stallions held all fourteen proteins, yet seven of the proteins were present in all samples, although the relative concentrations varied.

No research has been found that combines the characterization of the different seminal proteins with fertility rates. If this were to happen, the beneficial proteins could be isolated, characterized and used to develop (in conjunction with antibiotics) an extender that would replace the stallion's innate seminal plasma and increase the longevity and fertility of cooled semen.

To remove the seminal plasma the semen must be centrifuged. The number of studies on the effects of centrifugation of equine semen is limited. Two studies from Colorado Sate University in Fort Collins looked at the effects of centrifugation. One study examined seminal plasma removal after storage and found that centrifugation and partial removal of seminal plasma was not detrimental to stallions that were deemed "good coolers", but that it increased the progressive motility of "poor cooling" stallions after 48 hours of cooling and storage (6). The other study demonstrated that centrifugation prior to cooling was better than centrifugation after cooling and prior to freezing in terms of the progressive motility (7). If the effects are severely detrimental, the procedure needs to be re-evaluated to determine another method of separation. For example, scientists are currently evaluating the benefits of a cushion in the centrifugation tube to reduce the impact of sperm on the sides of the tube during spinning. This information is vital in the successful cooling and shipping of equine semen for artificial insemination.

Procedures and Methods

Collection occurred following the procedure outlined in Equine Reproduction (11). The Missouri model artificial vagina (AV) provided by Dr. Robert Kline and The Ohio State University was employed. Collection was done on three stallions' and sperm motility and count were evaluated via a microscope and densimeter respectively (11). The ejaculate was split into three separate groups. Two were centrifuged at 500g for 10 min, one of which had the removal of about 95% of the seminal plasma by a pipette as suggested by AAEP(1). The third tube was not centrifuged and the seminal plasma remained. All three were then diluted with EZ-mixin (with amacasin), resulting in 4.75 ml aliquots which placed in whirl-pak bags for a concentration of 25-50 million sperm per ml. Centrifugation and dilutions of the semen occurred within fifteen minutes of collection, as recommended (11). The semen was then cooled and stored in a refrigerator. After 24, 48, and 72 hours a portion of the semen was re-warmed (using a slide warmer) to 100 degrees C and motility was determined. The experiment was repeated using Ticarcillin, Polymixin B and Gentamycin to decrease any effect of extender on sperm motility/count. Motility was taken for all three samples at each time, from each horse and with each of the diluents. Results were recorded and statistically analyzed using a three group comparison and variability was plotted using SAS and Microsoft excel.

Results

Table 1. Percent motility for the stallion Zip.

Extender	Treatment	0 hrs	24 hrs	48 hrs	72 hrs
Ticarcillin					
	Centrifuged and diluted	80	40	50	45
	Diluted	90	30	25	10
	Centrifuged with the removal of seminal plasma and diluted	60	55	50	40
Amacasin					
	Centrifuged and diluted	70	5	10	5
	Diluted	70	80	50	20
	Centrifuged with the removal of seminal plasma and diluted	70	60	15	10
Gentamycin					
	Centrifuged and diluted	70	75	40	10
	Diluted	85	50	45	10
	Centrifuged with the removal of seminal plasma and diluted	70	30	5	25
Polymixin					
	Centrifuged and diluted	70	70	50	0
	Diluted	70	30	10	0
	Centrifuged with the removal of seminal plasma and diluted	60	40	0	5

Table 2. The percent motility determined for Cruise. The first set of data of two on this stallion.

Extender	Treatment	0 hrs	24 hrs	48 hrs	72 hrs
Ticarcillin					
	Centrifuged and diluted	80	40	45	40
	Diluted	90	50	30	5
	Centrifuged with the removal of seminal plasma and diluted	85	50	30	10
Amacasin					
	Centrifuged and diluted	90	40	0	5
	Diluted	70	30	50	40
	Centrifuged with the removal of seminal plasma and diluted	85	55	45	30
Gentamycin					
	Centrifuged and diluted	70	80	60	30
	Diluted	80	30	40	20
	Centrifuged with the removal of seminal plasma and diluted	50	30	5	25
Polymixin					
	Centrifuged and diluted	85	85	30	15
	Diluted	70	55	25	30
	Centrifuged with the removal of seminal plasma and diluted	50	5	10	15

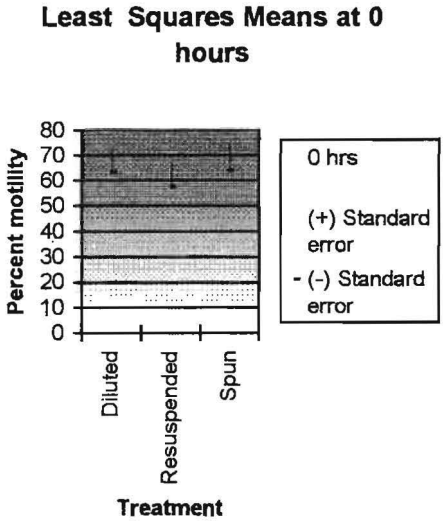
Table 3. The percent motility for the stallion Cruise, second set of data.

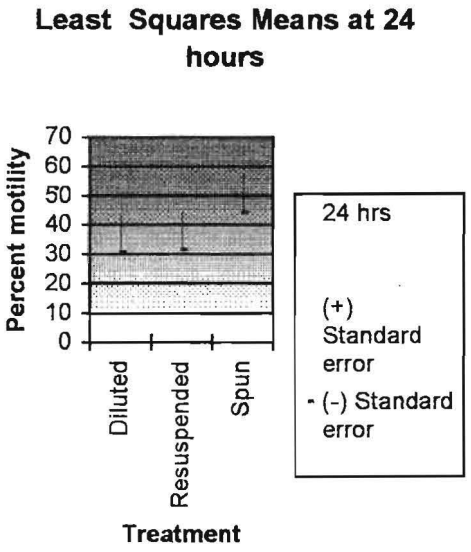
Extender	Treatment	0 hrs	24 hrs	48 hrs	72 hrs
Ticarcillin					
	Centrifuged and diluted	40	25	0	0
	Diluted	55	15	0	0
	Centrifuged with the removal of seminal plasma and diluted	30	10	0	0
Amacasin					
	Centrifuged and diluted	50	70	30	5
	Diluted	70	30	5	0
	Centrifuged with the removal of seminal plasma and diluted	70	50	10	0
Gentamycin					
	Centrifuged and diluted	50	70	0	0
	Diluted	50	25	0	0
	Centrifuged with the removal of seminal plasma and diluted	55	60	0	0
Polymixin					
	Centrifuged and diluted	70	10	0	3
	Diluted	20	20	0	0
	Centrifuged with the removal of seminal plasma and diluted	65	10	2	0

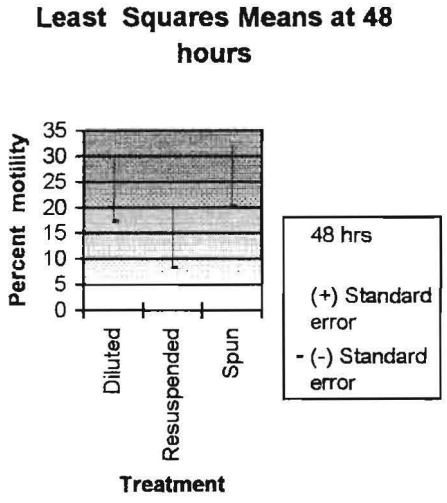
Table 4. Percent motility for the stallion Cowboy. This data was not used in the statistical calculations because it was incomplete.

Extender	Treatment	0 hrs	24 hrs	48 hrs	72 hrs
Ticarcillin					
	Centrifuged and diluted	NA	NA	NA	NA
	Diluted	NA	NA	NA	NA
	Centrifuged with the removal of seminal plasma and diluted	NA	NA	NA	NA
Amacasin					
	Centrifuged and diluted	55	0	0	0
	Diluted	75	0	0	0
	Centrifuged with the removal of seminal plasma and diluted	55	0	0	0
Gentamycin		50	25	20	15
	Centrifuged and diluted	NA	NA	NA	NA
	Diluted	20	10	0	0
	Centrifuged with the removal of seminal plasma and diluted				
Polymixin					
	Centrifuged and diluted	50	25	0	0
	Diluted	20	5	0	0
	Centrifuged with the removal of seminal plasma and diluted	40	0	0	0

Graphs 1-4. The following graphs are of the statistical information as a result of the SAS program. The graphs show the least square means for each time (at hour 0, hour 24, hour 48 and hour 72). The y-axis is the percent motile, while the x-axis shows for which treatment group the data is graphed. All data except for that from the stallion Cowboy was used. They show the relationship of the least squares means plus and minus one standard deviation across the different treatments







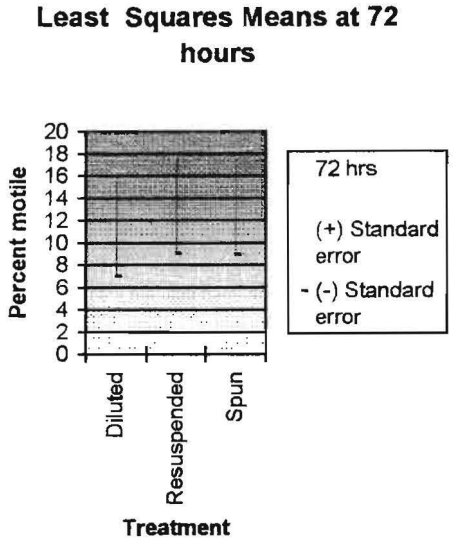


Table 5. The following table shows the p-diff values found using all stallions with the exception of Cowboy. These values compare the treatments to each other and are separated according to the different time periods.

	Diluted	Spun and resuspended	Spun and removal of seminal plasma
Treatment			
Time 0			
Diluted	---	0.4131	0.9531
Spun and resuspended	0.4131	---	0.3809
Spun and seminal plasma removed	0.9531	0.3809	---
Treatment			
Time 24			
Diluted	---	0.9285	0.1458
Spun and resuspended	0.9285	---	0.1710
Spun and seminal plasma removed	0.1458	0.1710	---
Treatment			
Time 48			
Diluted	---	0.2975	0.7336
Spun and resuspended	0.2975	---	0.1707
Spun and seminal plasma removed	0.7336	0.1707	----
Treatment			
Time 72			
Diluted	---	0.7321	0.7528
Spun and resuspended	0.7321	---	0.9781
Spun and seminal plasma removed	0.7528	0.9781	---

Discussion

The overall results of this experiment show that there is no difference between the three treatments of semen: that which was centrifuged with seminal plasma removed, that which was centrifuged without seminal plasma removal and that which was not centrifuged, although there are some trends that can be inferred. The stallion "Cowboy," was eliminated from the project because of an incomplete data set and therefore, the results include two sets of data from Cruise and one set of data from Zip.

The repeated measures analysis of variance test by SAS found the data within the subjects in regards to the different treatments to be insignificant ($p = 0.4530$). There was also no significant difference within the stallions in regard to type of extender used ($P=0.6515$). This could have arisen because as each stallion varies in their response to extenders, some stallions show no preference. It is important to keep in mind that these two stallions are a very small representative of the population and it is possible that they simply did not react differently to different extenders. In terms of variation between each of the subjects, there was again no significant difference in terms of treatment or type of extender used ($P=0.4234$ and $P=0.5879$ respectively).

Since the standard deviation lines cross in graphs one through four, it indicates that there is no significance between the treatments at this given time. As is shown, there is no time period in which there is a significant difference between the three treatments.

The two data points for motility at 24 and at 48 hours may indicate a scientific "trend". According to Dr. Shea Porr of The Ohio State University department of Animal Science, a "trend" may be indicated when the p-value is that of 0.1 (12). The twenty-four hour period, yielded a p-diff of 0.1458 in the comparison of the spun semen (with the

seminal plasma) to the diluted semen. Although this number is still greater than 0.1 it may indicate a trend which through more testing, might be shown as an important factor. This value implies that after 24 hours of cooling, spinning and removing the seminal plasma may increase the motility over semen that has only had diluent added.

A similar conclusion can be drawn at the 48 hour period. When comparing spun semen with the seminal plasma removed to the semen that is just spun the p-diff value is 0.1707. Further research may prove that there is a significant difference between semen that has had its seminal plasma removed when stored for 48 hours.

Because there is a potential trend in these stallions, it is possible that these stallions were both considered "poor" coolers. The problem is that the researchers never really define what a "good cooler" and a "poor cooler" are. The reason they might be categorized as "poor coolers" is that after forty-eight hours there was a possible difference between the semen from which the seminal plasma was removed and that which was simply spun, which parallels the 1999 study (6). The only problem with this is that there was no significance or trend between semen spun with the removal of seminal plasma and semen which was diluted ($p=0.7336$). It is possible that the seminal plasma has no effect on the motility or there is another underlying undetermined factor that affects the sperm motility. There was also no trend between semen which was spun and resuspended and semen which was diluted ($p=0.2975$). This implies that there is no effect of centrifugation on the motility at forty-eight hours which parallels the general conclusion of this project.

The study did show that there was a significant decrease over time in sperm motility. This is a logical conclusion because there is a progressive rate of death of the sperm over time.

Since there is no effect of centrifugation at 10,000g for 15 minutes on sperm motility, then there would be no problem with removing seminal plasma by this method. As centrifugation removes the seminal plasma and the motility is not decreased, there is no detrimental effects. This information is important in current studies looking at the role and effect of seminal plasma on sperm motility. By understanding that there are no detrimental effects by centrifugation then a portion of the differences in sperm motility can be attributed to differences in the characteristic seminal plasma from each stallion. As research in this area proceeds, centrifugation can be used as a safe and effective method of removing seminal plasma.

As suggestions for future research, the use of more stallions is required. It is common knowledge that there are significant differences between stallions' semen and their ability to cool and/or freeze well. By looking at a larger number of individuals perhaps the aforementioned trends will materialize into significant findings or will conclusively demonstrate no effect. Another aspect that would be of interest would be different centrifugation speeds and times. As it is vital to have the semen treated within about fifteen minutes of collection centrifugation time should be optimal to remove enough seminal plasma but still allow time for dilution and cooling (1). Centrifugation speeds may also play a role in sperm motility although there was not any research found dealing with this subject. How many g's can sperm tolerate and what is the most effective spinning rate for the successful cooling of semen? As it has been preliminarily determined that centrifugation does not affect sperm motility this technique should be refined to maximize sperm longevity.

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